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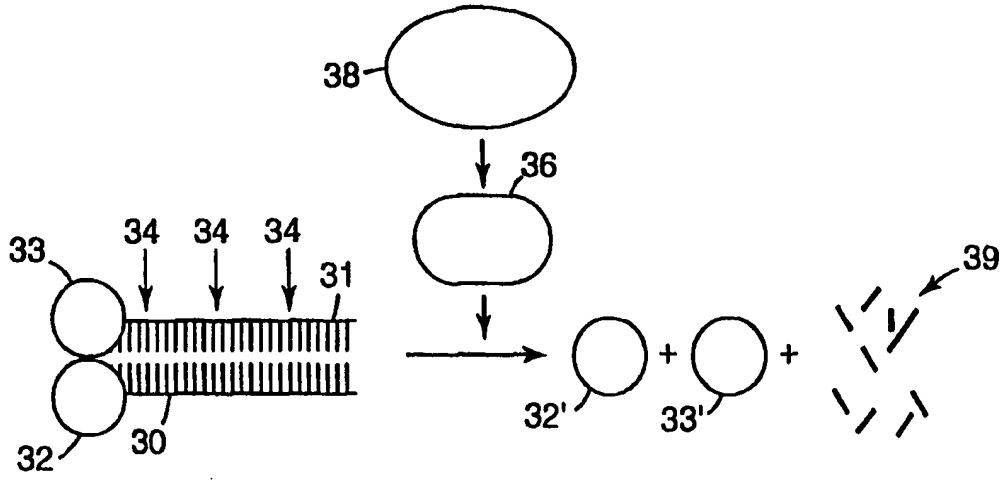

(57) Abstract

A reagent comprising an enzyme–specific cleavable polynucleotide (30, 31) substrate bearing quenched fluorescent moieties (32, 33) is provided, as is a method of making the same. The polynucleotide (30, 31) includes at least one fluorescent moiety (32) sufficiently close to another fluorescent moiety (33) to essentially quench fluorescence of the moieties (32, 33), wherein the fluorescent moieties (32, 33) become readily detectable by fluorometric techniques upon separation by cleaving the polynucleotide. A biological assay method is also provided wherein the reagent is combined with a test sample potentially containing the enzyme (36) being assayed wherein the enzyme (36) will cleave the polynucleotide to release the fluorescent moieties (32, 33), and produce an increase in fluorescence intensity. The assay method finds use in detection and identification of microorganisms, sterilization assurance, pharmaceutical discovery, enzyme assays, immunoassays, and other biological assays.

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Enzyme-Specific Cleavable Polynucleotide Substrate and Assay Method

TECHNICAL FIELD

This invention relates to a reagent comprising an enzyme-specific cleavable polynucleotide substrate bearing quenched fluorescent moieties, a method of making the same, and an assay method of using the same.

BACKGROUND OF THE INVENTION

Nucleases (DNases and RNases) are enzymes that hydrolyze, and cleave, polynucleotides, *i.e.*, nucleic acids, such as DNA and RNA. Such enzymes are present in every living cell to perform many essential functions in nucleic-acid metabolism, replication, recombination, repair, and modification. Nucleases can be differentiated by their substrate specificity: some cleave both DNA and RNA, others act on one type of nucleic acid only; some are sequence specific and others are not; some act on single-stranded nucleic acid, others require double stranded DNA to function. They may also be selective in, among other things, the location of the bond they cleave; their cellular location, *i.e.*, intra- or extracellular; ionic and pH requirements; and resistance to heat treatment. Nucleases are further explained in Linn, S.M., Lloyd, R.S., Roberts, R.J., *Nucleases* (2nd ed.), Cold Spring Harbor Laboratory Press, Plainview, NY, 1994.

Antibiotic resistant bacteria strains and food-borne illnesses are of significant concern to the public. The management of microbial risks in healthcare, cosmetics, food and beverage industries is a serious health and safety issue. Bacterial testing is an integral part of managing microbial risks. Enzymes produced by bacteria are frequently used to test for the presence of that bacteria. The ability of microorganisms to hydrolyze and cleave nucleic acids through production of nuclease has long been recognized. Enzymes are especially useful for detecting non-proliferating bacteria cells because the enzymes can catalyze a cleavage reaction with up to millions of substrate molecules per second, which

allows for detecting a small number of bacteria cells. Certain nucleases produced by bacteria constitute widely used means for identification and characterization of certain pathogenic species.

Known methods of measuring nuclease activity include UV absorption and electrophorectic or chromatographic separation of digested DNA fragments followed by fluorescent staining. Additional approaches have been developed that employ natural DNA, synthetic substrates and DNA-binding molecules:

(i) *Colometric DNA-binding molecules*. Toluidine blue (TB) or methyl green (MG), can be added to a mixture of natural DNA and an appropriate growth medium. The dye forms a complex with DNA that is either blue (TB) or green (MG). Hydrolysis of the complex by DNase releases the dye, producing a zone of color difference — rose pink (TB) or colorless (MG).

(ii) *Fluorescent DNA-binding molecules*. Acridine orange (AO), or other suitable dye such as Hoescht dye 33258 or dimeric cyanine nucleic acid stains such as those available under the trade marks TOTO™ or YOYO™ from Molecular Probes, Inc., Eugene, OR, can be added to a mixture of natural DNA and growth medium. These compounds are weakly fluorescent in aqueous solution, but become strongly fluorescent when bound to DNA. Hydrolysis of DNA by DNase causes complete removal of fluorescence, which can be seen as non-fluorescent halos around any bacterial colonies.

(iii) *Chromogenic synthetic substrates*. Two types of synthetic substrates are used to detect nuclease activities per this method. The first is Thymidine 5'-monophosphate p-nitrophenyl ester ammonium salt. It releases p-nitrophenol upon hydrolysis by a purified extracellular nuclease from a *Bacillus* species, as indicated by the increase in absorbance at 410 nm. The second is 5-bromo-4-chloro-indolyl-thymidine-3'-phosphate which, upon hydrolysis, releases the indoxyl group which produces a blue-green indigo color by autooxidation.

(iv) *Fluorogenic synthetic substrates*. There are two types. The first is a fluorometric method employing poly(deoxy(etheno)adenylate) — a derivative of poly(deoxyadenylate). This polynucleotide is weakly fluorescent when intact. However, upon DNase hydrolysis,

fluorescence enhancement may be observed (Excitation=320 nanometers (nm) and Emission=410 nm), as a result of release of fluorescent ethenomononucleotide. The signal may be continuously monitored and can be correlated to the amount of liberated mononucleotide. Another fluorometric method is known that makes use of the fluorescence "dequenching" technique. A 14-mer polynucleotide can be labeled with fluorescein 5-isothiocyanate (Ex=490 nm and Em=520 nm). Upon hybridization with an unlabeled complementary strand, 50% of the fluorescence is quenched due to interactions between DNA and fluorophores. Cleavage, *i.e.*, chain scission, of the hybridized species by a restriction endonuclease produces a 2-fold increase in fluorescence intensity due to "dequenching."

The *DNA-binding molecules* are usually not sequence specific, and signals may be affected by any endogenous DNA present in samples. In some cases, they may also be affected by the presence of surfactants, EDTA, or cell debris. *Chromogenic synthetic substrates* are usually required in large quantities (millimolar amounts), which may increase assay costs and in some cases lead to insolubility. Also, absorption-based methods are inherently several orders of magnitude less sensitive than fluorescence-based methods. As to *fluorogenic substrates*, there are few reagents available for nucleases. Additionally, reported methods either have moderate performance or are limited to specific enzymes, *e.g.*, exonuclease.

A fluorescence detection method is among the most sensitive detection techniques available today. Zeptomolar (10^{-21} M) amounts of enzyme molecules have been studied using a fluorescence microassay. Single enzyme molecules have been detected in an oil-dispersed droplet by fluorescence microscopy. Individual molecules of an enzyme have been manipulated electrophoretically in a capillary tube and monitored by fluorescence spectroscopy. See Xue, Q., Yeung, E.S., *Differences in Chemical Reactivity of Individual Molecules of an Enzyme, Nature*, 1995, 373, 681-683. Assays for detection of coliform bacteria using β-galactosidase as a marker enzyme have been developed. Fluorometry was found to have a 250-fold increase in sensitivity and 5 hour reduction in the time of detection, relative to colorimetric methods. See Van Poucke, S.O., Neils, H.J., *Development of a Sensitive Chemiluminometric Assays for the Detection of* β-

Galactosidase in Permeabilized Coliform Bacteria and Comparison with Fluorometry and Colorimetry, Appl. Env. Microbiol., 1995, 61, 4505-4509.

DISCLOSURE OF THE INVENTION

Briefly, this invention provides a reagent comprising at least one enzyme-specific cleavable polynucleotide substrate bearing at least one fluorescent moiety in close proximity to at least one other adjacent fluorescent moiety, the adjacent fluorescent moieties quenching fluorescence in each other, the fluorescent moieties being readily detectable by fluorometric techniques upon cleavage of the substrate.

The reagent may comprise an enzyme-specific cleavable polynucleotide substrate comprising a self-complementary single strand polynucleotide having 3'and 5' termini each bearing a fluorescent moiety; a polynucleotide substrate comprising two complementary hybridized polynucleotide strands each having 3'and 5' termini wherein each terminus of one or both 3'/5' pair(s) bears a fluorescent moiety; or a polynucleotide substrate comprising a single strand polynucleotide bearing along its length at least two pendent adjacent fluorescent moieties.

This invention also provides a method of biological assay comprising the step of:

combining (1) a reagent comprising at least one enzyme-specific cleavable polynucleotide substrate bearing at least one fluorescent moiety in close proximity to at least one other adjacent fluorescent moiety, said adjacent fluorescent moieties quenching fluorescence in each other, but being readily detectable by fluorometric techniques upon separation, and (2) a test sample potentiallly containing the specific enzyme being assayed, wherein the presence of the specific enzyme being assayed will result in cleavage of the polynucleotide, separation of the fluorescent moieties, and an increase in fluorescence intensity.

A further embodiment of this method comprises the additional step of measuring the increase in fluorescence intensity.

The fluorescence may readily be observed by conventional techniques such as radiant energy illumination, a fluorescent microscope, a 96-well plate reader, or a flow cytometer.

The invention further provides a method for making the enzyme-specific cleavable polynucleotide reagent bearing quenched fluorescent moieties. This method comprises making a reagent comprising an enzyme-specific cleavable polynucleotide bearing fluorescent moieites that quench each other through dye dimerization and that are detectable by fluorometric techniques upon separation, said method comprising the step of:

combining one or more fluorescent compounds bearing one or more fluorescent moieties and one or more reactive groups with enzyme-specific cleavable polynucleotides selected from the group consisting of (1) self-complementary single strand polynucleotides having one or more end groups reactive with one or more of the fluorescent compounds, (2) single strand polynucleotides having within the polynucleotide, at locations other than the termini, two or more moieties having pendent groups reactive with one or more of the fluorescent compounds, and (3) complementary polynucleotides each strand having at least one reactive end group at the 3' and/or 5' termini, respectively, of one or both 3'/5' pair(s) which react with one or more of the fluorescent compounds, such combination taking place under reactive conditions to produce said reagent.

In this description:

"dye dimerization" means formation of a non-covalently-bonded complex between two dye groups;

"dye stacking" means formation of a non-covalently-bonded complex between two or more dye groups, two complexed dye groups are referred to as a "dimer" and three complexed dye groups are referred to as a "trimer";

"enzyme-specific cleavable polynucleotide" means a linear sequence of nucleotides comprising specific bonds that are subject to cleavage by specific enzymes;

"fluorescence" means light emission by a substance at a given wavelength upon absorbing light of a different wavelength, wherein light emission occurs only during light absorption;

"fluorescent quantum yield" means the ratio of the number of fluorescent photons emitted by an emitting substance to the total number of photons absorbed by the substance;

"fluorogenic substrate" means an essentially non-fluorescent material acted upon by an enzyme to produce a fluorescent compound;

"fluorophore", "dye moiety," or "dye group" means a molecule or portion of a molecule that emits light at a given wavelength when stimulated by absorption of light of a different (usually shorter) wavelength.

This invention provides advantages over conventional assay methods for detection and identification of microorganisms. It provides a rapid and convenient approach that employs enzyme-specific cleavable polynucleotide substrates having fluorescent moieties, also referred to herein as fluorogenic nuclease substrates, for measuring activities of and/or detecting the presence of extracellular and intracellular enzymes. It can also be adapted to sequence-dependent or sequence-independent tests. The method and reagents of the invention have led to improved accuracy, faster detection, and overall lower cost in detection and identification of microorganisms. In various embodiments, the invention employs the use of measurable differences in fluorescence to allow detection of nuclease. In preferred embodiments, the present invention provides fluorogenic indicators that show a higher signal level when cleaved compared to the much lower signal level when quenched and that operate preferably in the visible wavelength range to minimize interference due to growth medium background fluorescence.

The present invention allows more accurate and sensitive measurement of the presence of nuclease by, among other factors, red-shifting the emission wavelength from far UV region (about 350 to 400 nanometer) to the 500-600 nm region of the electromagnetic spectrum and reducing the effect of background signal levels of intact reagents. Another advantage of the present invention is that it is a homogeneous biological assay method, which requires no developing agent. Therefore, it does not require time-consuming separation steps as in assay formats such as enzyme-linked immunosorbent assays (ELISA) and bioluminescence. Detection and identification of microorganisms can be performed by using the primary isolation medium.

As can be seen, the present assay method features more efficient use of reagents, labor, time, and equipment. It also offers the potential of examining several bacteria and/or enzymes simultaneously through the judicious design and choice of polynucleotides and the fluorophores attached thereto. Assay methods described in this invention have implications in the detection and identification of microorganisms, sterilization assurance, pharmaceutical drug discovery, clinical assays, nucleic acid hybridization, sequencing and amplification.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic illustration of a conceptual mechanism of a fluorogenic nuclease substrate being cleaved by a bacteria-produced enzyme wherein the fluorogenic nuclease substrate is a self-complementary single strand polynucleotide exhibiting intramolecular dimerization and quenching of fluorescent moieties attached at the 3' and 5' termini of the polynucleotide, which fluorescent moieties become separated and readily detectable upon cleavage of the polynucleotide.

Fig. 2 is a schematic illustration of a conceptual mechanism of a fluorogenic nuclease substrate being cleaved by a bacteria-produced enzyme wherein the fluorogenic nuclease substate is a single strand polynucleotide having pendent fluorescent moieties in sufficiently close proximity to quench each other through dye stacking, which fluorescent moieties become separated and readily detectable upon cleavage of the polynucleotide.

Fig. 3 is a schematic illustration of a conceptual mechanism of a fluorogenic nuclease substrate being cleaved by a bacteria-produced enzyme wherein the fluorogenic nuclease substrate comprises two complementary hybridized polynucleotide strands exhibiting intermolecular dimerization and quenching of two fluorescent moieties labeled at the 3' terminus and 5' terminus, respectively, of the two complementary polynucleotides, which fluorescent moieties become separated and readily detectable upon cleavage of the polynucleotide.

Fig. 4 is a graph of the relative fluorescence intensity of a tetramethylrhodamine-labeled 25-mer double stranded polynucleotide before (line b) and after (line a) incubation with micrococcal nuclease in a 100 millimolar pH 8.9 bicarbonate buffer at 37°C.

Fig. 5 is a graph of the absorption spectra of a tetramethylrhodamine-labeled 24-mer self-complementary single strand polynucleotide before (line c) and after (line d) incubation with micrococcal nuclease in 100 millimolar pH 8.9 bicarbonate buffer solution at 37°C.

DETAILED DESCRIPTION

In this invention, a novel approach to provide a reagent comprising a nuclease substrate is described. The substrate provides for separation of quenched fluorescent moieties upon exposure to the nuclease being assayed thereby causing a detectable increase in fluorescence.

The nuclease substrate is made by first specifying a polynucleotide to be used. This is done by selecting the bases or base pairs (A/T or G/C) to be included in the polynucleotide. Any configuration that will bring the fluorescent moieties into close proximity will work. Hybridization may be used to achieve a suitable configuration. Hybridization occurs as complementary base pairs of polynucleotide(s) are attracted to each other. Possible configurations include, for example, a self-complementary single-strand polynucleotide having fluorescent dye moieties attached at both the 3' and 5' ends that will self-hybridize, *i.e.*, fold over, to bring fluorescent moieties attached at each end of the polynucleotide into close proximity. An alternative configuration comprises two mutually complementary single strand polynucleotides each having 3'and 5' termini wherein each terminus of one or both 3'/5' pairs bears a fluorescent moiety which polynucleotides hybridize to form a double stranded polynucleotide thereby bringing fluorescent moieties attached at one or both ends of each single strand polynucleotide into close proximity. Another method involves a single strand polynucleotide wherein two or more fluorescent dye moieties are pendent from the polynucleotide chain and are spaced sufficiently closely along the chain at

appropriate lengths to cause the fluorescent moieties to come into close proximity and quench fluorescence.

For a hybridized configuration, the extent of hybridization need only be sufficient to bring the attached dye moieties into close proximity. Typically, hybridization of approximately the five base pairs closest to the 3'/5' termini to which the fluorescent moieties are attached will suffice. The number may vary depending on factors such as the type of base pairs in the polynucleotide and the dimerization constant of the fluorescent moieties.

The polynucleotide is further specified to contain one or more bonds that are cleavable by the nuclease sought to be assayed. The bond(s) are strategically located within the polynucleotide so that cleavage will cause separation of the fluorescent moieties. For a self-complementary single strand polynucleotide or two mutually complementary polynucleotides that form a double strand polynucleotide, the cleavable bond(s) should typically be located near the end or ends of the polynucleotide where the fluorescent moieties are attached. For a single strand polynucleotide having pendent moieties, the cleavable bonds should typically be located between the dye moieties.

Bonding sites for the selected fluorescent moieties are also incorporated into the polynucleotide at the specific locations where the fluorescent moieties are desired. These sites may be at the ends (termini) of the polynucleotide as in the case of hybridized polynucleotides, or along the polynucleotide strand, *i.e.*, backbone, as in the case of a single strand polynucleotide to which pendent dyes will be attached. These bonding sites comprise reactive groups such as aliphatic primary amines that will readily react with the fluorescent compounds comprising fluorescent moieties to be attached to the polynucleotide.

Once the polynucleotide has been specified, it can be synthesized, for example, by known manual or automated DNA synthesis methods. The polynucleotide is then reacted with one or more compounds comprising fluorescent moieties. The fluorescent compounds typically also comprise reactive groups which will react with the reactive groups on the polynucleotide. The reaction takes place under appropriate concentration, agitation, pH, ionic, and temperature conditions which will cause the fluorescent moieties to attach to the polynucleotide

at selected sites. The particular necessary conditions vary based on the reactive groups present in the polynucleotide and fluorescent compound being used, respectively. The pH is controlled by using a buffer solution such as a carbonate or bicarbonate buffer solution. The fluorescent moieties used in this invention possess the capability to dimerize or stack to cause quenching of fluorescence.

This invention also provides a method of biological assay comprising the step of:

combining (1) a reagent comprising at least one enzyme-specific cleavable polynucleotide substrate bearing at least one fluorescent moiety in close proximity to at least one other adjacent fluorescent moiety, said adjacent fluorescent moieties quenching fluorescence in each other, but being readily detectable by fluorometric techniques upon separation, and (2) a test sample potentially containing the specific enzyme being assayed, wherein the presence of the specific enzyme being assayed will result in cleavage of the polynucleotide, separation of the fluorescent moieties, and an increase in fluorescence intensity.

A further embodiment of this method comprises the additional step of measuring the increase in fluorescence intensity. This can be done with radiant energy illumination, a fluorescent microscope, a 96-well plate reader, or a flow cytometer.

Figure 1 depicts a preferred embodiment of the invention. Polynucleotide 10 can be synthesized so as to be self-complementary, that is, it undergoes intrastrand self-hybridization. When fluorescent dye moieties 11 and 12 are attached at the 3'- and 5'- termini, only weak fluorescence is observed because the dye moieties are in sufficiently close proximity to cause mutual quenching. Upon treatment with nuclease 13, generated by bacteria 14, polynucleotide 10 may be cleaved at sites such as, *e.g.*, 15, 16, and 17, which can destroy the self-hybridized structure to produce nucleotide fragments 18 and non-dimerized dye moieties 11' and 12', which disassociate and fluoresce freely. In this embodiment, the polynucleotide chain must be of sufficient length to allow the polynucleotide to fold over and bring the fluorescent dye moieties into close proximity. Typically, approximately fifteen base pairs is sufficient.

Figure 2 depicts a second preferred embodiment of the invention. A plurality of fluorescent dye moieties 22 are attached to polynucleotide 20 which contains various sites 24 that are susceptible to enzymatic cleavage. The dye moieties 22 are close enough to each other so that fluorescence of dye moieties 22 is quenched. The conjugate (comprising polynucleotide 20 and fluorescent dye moieties 22) can be treated with nuclease 26, generated by bacteria 28, to cleave polynucleotide 20. Cleavage produces nucleotide fragments 29 and allows dye moieties 22' to separate from one another in the medium such that they fluoresce freely and are easily detectable. In this embodiment, it is preferred to have the fluorescent moieties 22 separated by less than 10 nanometers measured base to base along the backbone of the polynucleotide. This length is determined by indirect methods based on the known or estimated lengths of polynucleotide bases. A configuration as shown in Figure 2 could be used to test for multiple types of bacteria or enzymes by having a series of different types of paired or grouped fluorescent moieties, each type distinguishable from the others using fluorometric techniques, with the individual moieties of each pair or group being joined by, or on opposite sides of, a cleavable bond susceptible to cleavage by a different bacterial enzyme.

Figure 3 depicts yet another preferred embodiment of the invention. Complementary hybridized polynucleotides 30 and 31 have fluorescent dye moieties 32 and 33 attached at their 3'- and 5'- termini, respectively. Nuclease 36, generated by bacteria 38, cleaves polynucleotide(s) 30 and/or 31 at cleavage sites 34 to produce nucleotide fragments 39 and non-dimerized dye moieties 32' and 33' that are easily detectable. A further embodiment could comprise dye moieties attached to each 3' and 5' termini of each complementary polynucleotide. A configuration as shown in Figure 3 could be used to test for two types of bacteria or enzymes at the same time by labeling each complementary 3'/5' termini pair with a different type of fluorescent moiety, each type distinguishable from the other using fluorometric techniques, and constructing the double-stranded polynucleotide to have cleavable sites near each terminus of the double-stranded polynucleotide susceptible to cleavage by a different type of bacterial enzyme.

Fluorogenic substrates are molecules that change from essentially nonfluorescent to highly fluorescent upon enzymatic hydrolysis. They are widely used as molecular probes for studies and tests of viral and bacterial enzymes such as proteases, nucleases, saccharidases, phosphatases and kinases. The fluorescence of the substrate may be readily observed by conventional techniques such as radiant energy illumination, a fluorescent microscope, a 96-well plate reader, or a flow cytometer.

The enzyme-specific cleavable polynucleotide substrates bearing fluorescent moieties, or fluorogenic nuclease substrates, useful in the present invention can be made by chemical reaction of a polynucleotide and fluorescent dyes such as fluorescein and rhodamine that will dimerize or stack. Useful polynucleotides are commercially available (*e.g.*, from GeneMed Biotechnologies, South San Francisco, CA). To prepare the reagents of the invention, two or more fluorescent moieties are bonded to the polynucleotide so that dimerizing or stacking of the fluorescent moieties can occur (*e.g.*, through substantial hybridization, proximity, interstrand hybridization, intrastrand hybridization, etc.) so as to mutually quench (referred to as "self-quenching" when the fluorescent moieties are identical) the fluorescence of the fluorescent moieties.

Fluorescence dye quenching may take place by any of a number of known mechanisms, including energy transfer and dye dimerization. In these cases, when a fluorescent dye molecule or moiety is excited by input of energy, typically by irradiation with a specific wavelength of light, energy is transferred from the dye moiety to another moiety, rather than being dissipated by fluorescence. Energy transfer, also referred to as Förster-type dipole-dipole interaction, occurs between a fluorescent donor and an acceptor, which may or may not be fluorescent. Energy transfer generally takes place when the distance between donor and acceptor along the substrate backbone is on the order of 10 nanometers. Through energy transfer, the fluorescence of the donor moiety is quenched. Dye dimerization or dye stacking, on the other hand, occurs when two or more fluorescence moieties are close enough to each other to allow their planar aromatic rings to interact to form ground state complexes such as dimers and trimers. Each fluorescent moiety acts toward the other fluorescent moiety(ies) causing mutual quenching. The

quenching. The absorbance spectra of dyes in a dimer- or stacked state are substantially different from those of the same dyes in energy transfer pairs. Dye dimer absorption spectra show a characteristic decrease in absorbance of the principal absorption peak as dye concentration increases, while showing a characteristic increase in absorbance of the shoulder peak. This phenomenon is illustrated in Figure 5. For the dye molecules in the graph of Figure 5, the principal absorbance peak occurs at approximately 560 nm and the shoulder peak occurs at approximately 530 nm. The dimerized sample (c) exhibits absorbance peaks of approximately 0.03 absorbance units (AU) for both the principal absorbance peak and the shoulder peak while the undimerized sample (d) shows a principal absorbance peak of approximately 0.06 AU and a flattened shoulder peak of approximately 0.028 AU. The phenomenon occuring in the dimerized sample is commonly referred to as "band splitting." Dimerization or stacking takes place through the formation of ground-state complexes (*i.e.*, through close physical proximity), whereas energy transfer interactions occur through space. Therefore, spectral changes characteristic of dye dimerization are not seen for dyes that interact by energy transfer mechanisms.

The quenching mechanism of the dimerized or stacked dye pairs or dye groups used in the instant invention is distinct from the quenching mechanism of fluorescence energy transfer donors and acceptors. The type of dyes that exhibit dimerization or stacking characteristics when bonded to the polynucleotide within a sufficiently close proximity to one another include those dyes which have a generally planar aromatic structure so as to be capable of forming homo- or heterodimers when in solution at sufficiently high dye concentrations (for example, 10^{-2} to 10^{-4} M). For many applications this phenomenon is obviously undesirable, but is used to advantage in the present invention. Concentration increases can be accomplished either by increasing the amount of dye in a unit volume or by physically locating two (or more) dye moieties closely together, for example, on a polynucleotide or other small molecule. When the dye moieties are brought into close proximity this causes an effective increase in the local concentration and the polynucleotide bearing fluorescent moieties will stay quenched regardless of the total concentration.

Planar aromatic dyes of the fluorescein and rhodamine families, such as fluorescein, tetramethylrhodamine (TMR), Rhodamine B, and X-rhodamine are representative dyes that will dimerize in sufficiently high concentration. Other suitable fluorescent dyes include, *e.g.*, cyanines, and boron-heterocyclic dyes such as 4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indacene available under the trade mark BODIPY™ from Molecular Probes, Inc. of Eugene, OR. Dyes of the rhodamine family such as tetramethylrhodamine, X-Rhodamine, and Rhodamine B have very high fluorescent quantum yields (approximately 0.85) in the visible wavelength range (400 - 700 nm). For this reason they are frequently used for detection of minute amounts of substances.

The dye moieties used in this invention additionally comprise reactive end groups which facilitate the attachment of the fluorescent moiety to the polynucleotide. These end groups may comprise succimidylester, maleimide, esters, thioisocyanates, isocyanates, and iodoacetimide. Dye molecules with these reactive groups already attached are available from, for example, Molecular Probes, Inc. (Eugene, OR)

The polynucleotides used in this invention must have one or more enzyme-specific cleavable sites. Upon cleavage of the fluorescence-quenched polynucleotide, which bears two or more fluorescent moieties, fluorescence intensity will be enhanced as a result of dissociation of the dimerized or stacked fluorescent moieties. This allows for easy detection of the fluorescent moieties thus indicating the presence of specific enzymes and, if applicable, the presence of the bacteria that produced the enzyme. Due to the interaction between transition dipoles of the resonating dimeric or stacked structure, the fluorescent quantum yield of the dimeric or stacked structure will be quite low when the polynucleotide is intact. When the dimeric or stacked stucture dissociates after enzymatic cleavage of the polynucleotide, the fluorescent moieties can become separated so that significantly higher fluorescence quantum yield in aqueous solution will be observed due to high intrinsic fluorescence. In this manner, the increase in fluorescence and the presence of the hydrolyzing enzyme can be discerned. On this basis, a homogenous enzyme assay, requiring no developing agent and no separation step, can be designed wherein enzyme-specific cleavable

polynucleotides bearing fluorescent moieties are placed in solution and an enzyme analyte is added so that any enzyme present in the analyte cleaves the polynucleotide causing dimerization or stacking to decrease with an attendant increase in fluorescence. Therefore, enzyme activity can be directly related to the net increase in fluorescence intensity. If the enzyme is secreted from actively metabolizing cells, the fluorescence intensity may be related to the metabolic activity of the cells.

The ability of one enzyme molecule to turn over millions of reagent molecules is an amplification process. This amplification is further enhanced when the enzyme is from a live cell culture because more enzyme molecules are generated as the cells grow. These two factors, when coupled with fluorescence techniques, offer a promising approach to rapid, sensitive, and specific detection of bacteria.

Representative polynucleotides useful to produce fluorogenic nuclease substrates must have the requisite chemical bond attacked by the nuclease being assayed. For example, as discussed below, micrococcal nuclease is known to hydrolyze polynucleotides at phosphodiester bonds, so any polynucleotide with this characteristic can be a micrococcal substrate.

Chemical modifications of polynucleotides are accomplished using nucleotide analogs and DNA synthesis reagents. For example, phosphoramidites containing aliphatic primary amines may be introduced into a polynucleotide chain at desired positions through automated DNA synthesizers such as the Nucleic Acid Synthesis and Purification System (Model # ABI 3948, Perkin-Elmer Applied Biosystems, Foster City, CA). Terminus modifiers and amino-modified-deoxythymidine (dT) are commercially available to introduce an aliphatic amine with a three-carbon, six-carbon, or nine-carbon linking moiety at the 3' and/or 5' end of polynucleotides. The amine can then react with a variety of labels such as biotin, fluorescent dyes, or alkaline phosphatase to form polynucleotide conjugates. Common applications of these modified polynucleotides include: (i) nonradioactive hybridization probes; (ii) sequence specific cleavage of DNA, (iii) automated DNA sequencing, and (iv) affinity chromatography. Other moieties that can be added to the ends of a polynucleotide to react with the dye moieties to

be attached include those with reactive H groups such as free thiol (SH), carboxyl groups, and OH groups. Amines and thiols are preferred.

Enzymatic cleavage is achieved by contacting the fluorogenic nuclease substrate, *i.e.*, the reagent, with the specific enzyme being assayed. Target enzymes suitable for use in the present invention include all enzymes generally classified as nucleases, *i.e.*, those that hydrolyze nucleotide bonds, including, for example, thermonuclease or *Staphylococcus* nuclease.

When the enzyme is intracellular, the enzyme may be made available to contact with the fluorogenic nuclease substrate by the use of agents that increase the permeability of the outer membrane (OM) of the enzyme-carrying bacteria. Ethylenediaminetetraacetic acid (EDTA) is commonly used for this purpose by affecting the outer membrane barrier of gram-negative enteric bacteria. Under certain conditions, the OM becomes ruptured and the intracellular enzymes can be released.

For rapid and sensitive tests, it is important to maximize the signal-to-noise ratio, *i.e.*, maximize the desired signal over undesired background signals such as, in this case, autofluorescence of bacteria and growth medium. Most traditional fluorogenic substrates emit in the far UV wavelength region (350-450 nm) where most bacteria growth media have significant autofluorescence. For example, a typical culture medium for *Staphylococcus aureus* has two significant emission maxima at 425 nm and 475 nm when excited at 360 nm. In order to avoid this problem, high substrate concentrations are normally used. This can result in higher assay cost, higher toxicity to organisms, and, sometimes, precipitation of substrate. The present invention overcomes the difficulty of autofluorescence interference by red-shifting the detection wavelength to the visible spectrum through the use of fluorescent dyes that emit at wavelengths much longer than those of background fluorescence (*e.g.*, tetramethylrhodamine: Ex = 550 nm, Em = 580 nm). Moreover, the concept described in this invention can be applicable to other dyes that will also red-shift the emission to even longer wavelengths. Typically, useful dyes may have the following characteristics: high extinction coefficient (>80,000 $cm^{-1}M^{-1}$), high quantum yields (>0.85 in aqueous solution), spectra that are insensitive to

solvent and pH, aqueous solubility, photostability, and a dimerization constant preferably greater than 10^3.

A number of staphylococci strains show DNase activity. An enzyme historically used for identification purposes is the extracellular DNase of *Staphylococcus aureus (S. aureus)*. *S. aureus* nuclease is excreted in great quantities, is heat-stable, and requires Ca^{2+}, but not Mg^{2+}, for activity. These characteristics are used for differentiating *S. aureus* from other staphylococci for detection of staphylococcal contamination in foods. Extracellular DNase is also produced by *Streptococcus* species, *Serratia* species, and *Bacillus* species.

Micrococcal nuclease, an extracellular enzyme from *Staphylococcus aureus*, is used as a model system to demonstrate utility of this invention. This enzyme cleaves the 5' phosphodiester bond of a polynucleotide strand, remains stable and active at elevated temperatures (>60°C), and is frequently used as an indicator for *S. aureus*. The presence of nuclease activity at 60°C is a confirmative test for *S. aureus*. Micrococcal nuclease does not require a cofactor for its function — a feature that can reduce assay complexity and cost.

In summary, this invention provides novel nuclease reagents, an assay method for using the reagents, and a method for making the reagents. The reagents comprise single or double stranded synthetic polynucleotides bearing two or more fluorescent moieties which may be dye molecules (*e.g.* tetramethylrhodamine). Fluorescence enhancement after nuclease hydrolysis and cleavage is between 2 and 6 fold, depending on the specific configuration of the reagent. These reagents, when coupled with the "double cascade" phenomenon in which each enzyme molecule is capable of turning over millions of reagents molecules and more enzyme molecules are released as bacterial cells divide and grow exponentially, offer promising approaches to rapid, sensitive and specific detection of bacteria. This invention finds use in, but is not limited to, detection and identification of microorganisms, sterilization assurance, pharmaceutical discovery, enzyme assays, and immunoassays.

Objects and advantages of this invention are further illustrated by the following examples, but the particular materials and amounts thereof recited in

these examples, as well as other conditions and details, should not be construed to unduly limit this invention.

EXAMPLES OF THE INVENTION

Test Methods

Fluorescence Spectra

All fluorescence measurements were carried out in a spectrofluorometer available under the trademark FLUOROLOG™ from Spex Instruments of Edison, NJ. In a typical measurement, 3 ml of assay solution was placed in a quartz cuvette and the cuvette placed in a sample holder of the spectrofluorometer. Fluorescence emission spectra of the assay solution was then measured at a fixed excitation wavelength at room temperature. The excitation and emission slit widths of the spectrofluorometer were both set at 0.5 millimeter. Fluorescence spectra of the assay solution both with and without the presence of nuclease were measured under the same instrumental conditions.

Example 1

In this example, two tetramethylrhodamine (TMR) dye moieties were bonded to the 3' and 5' termini of a self complementary 24-mer polynucleotide. Intrastrand hybridization brought the dye moieties into close contact with each other and therefore quenched fluorescence. Enzymatic cleavage at multiple sites resulted in the destruction of the ordered structure. The dye moieties then became undimerized, producing a significant increase in fluorescence. This concept is schematically illustrated in Figure 1.

A self-complementary polynucleotide, $NH_2CH_2CH_2CH_2$-5'-**AAC AAA GGA TAA TTA TCC TTT GTT**-3'-$CH_2CH_2CH_2NH_2$, was obtained from GeneMed Biotechnologies, Inc. (South San Francisco, CA) and its chemical structure was confirmed by DNA sequencing. About 270 nanomoles (nmoles) of the above polynucleotide was dissolved in 600 µl of deionized (D.I.) water. 90 nmole (200 µl) of the polynucleotide solution was reacted at room temperature with 4 mg of tetramethylrhodamine (TMR) succimidylester (obtained from Molecular Probes,

Inc., Eugene, OR), which had been pre-dissolved in one drop of dimethylsulfoxide (DMSO), in 100 millimolar (mM) pH 8.3 sodium bicarbonate buffer. The mixture was of a sufficient volume to maintain the proper pH -- a volume of approximately 2 ml. In this amidation process, the ester of the TMR moiety reacted with the amine moiety attached to the polynucleotide.

The reaction mixture was introduced from a pipet into a size-exclusion column (Model PD-10, Pharmacia Biotech, Inc., Piscataway, NJ) containing epichlorohydrin-crosslinked (alkaline conditions) dextran gel, a packing material, available under the trademark SEPHADEX™ G25 from Pharmacia Biotech, Inc., to separate labeled polynucleotide from the unreacted dye. The reaction mixture was passed through the column by force of gravity. The DNA fraction was further purified by high performance liquid chromatography (HPLC) on a C18 reversed phase column (Model 600E HPLC, Waters Corp., Milford, MA). The mobile phase in the column was a gradient of acetonitrile (ACN) and 0.1 M triethylamine acetate (TEAA) in water in which the volume ratio of TEAA to ACN started at 92:8 and linearly decreased to 8:92 over 30 minutes. The resultant conjugate had the above polynucleotide structure with the addition of a TMR moiety on each aliphatic amine linking moiety at the 3' and 5' termini.

HPLC fractions containing the purified conjugate were pooled, frozen in dry ice, and then vacuum dried at room temperature. The dry conjugate was then dissolved in 5 ml of 100 mM pH 8.9 sodium carbonate buffer solution. As shown in Figure 5, the absorption spectra measured by use of a spectrophotometer (Model MPC-3100, Shimadzu Scientific Instruments, Columbia, MD) showed band-splitting characteristic of dimerized tetramethylrhodamine. A small amount (about 0.5 mg) of micrococcal nuclease (obtained from Sigma Chemical Co., St. Louis, MO) was added to 3 ml of the above buffered solution. The mixture was incubated overnight at 37°C for enzymatic hydrolysis of the 5'-phosphodiester bonds of the polynucleotide bearing fluorescent moieties. The solution was then cooled to room temperature. A FLUOROLOG™ spectrofluorometer was then used to measure the fluorescence spectra at room temperature. Enzymatic cleavage resulted in a relative enhancement in fluorescence intensity from 12000 to 28000 at 580 nm and an increase in absorbance

from 0.03 absorbance units (AU) to 0.06 AU at 560 nm when excited with light at 520 nm.

In another test, the length of the aliphatic amine linking moiety at the 5' terminus was increased to allow more efficient stacking between the two dye moieties. The following polynucleotides with longer aliphatic amine linking moieties were obtained from GeneMed, labeled and tested under conditions similar to those previously stated in this example.

H_2N-$(CH_2CH_2O)_6$-$(CH_2)_3$-5'-**AAC AAA GGA TTA TAA TCC TTT GTT**-3'-$CH_2CH_2CH_2$-NH_2

H_2N-$(CH_2CH_2O)_9$-$(CH_2)_3$-5'-**AAC AAA GGA TTA TAA TCC TTT GTT**-3'-$CH_2CH_2CH_2$-NH_2

A two-fold enhancement of fluorescence was observed in each case.

Example 2

In this example, pendent tetramethylrhodamine dye moieties were attached at multiple positions within a polynucleotide sequence. Because of close proximity, the dye moieties stacked together to quench fluorescence. Enzymatic cleavage of the polynucleotide at multiple sites disintegrated the conjugate to liberate dye moieties from a dimeric or stacked state to single moieties, producing a concurrent increase in fluorescence intensity. This concept is schematically illustrated in Figure 2.

The polynucleotide strand was designed to contain a total of four amino groups in alternate bases as follows: 5'- TTT TTT **Z** T **Z** T **Z** T **Z** TTT TTT -3', where **Z** was an amine-modified C_6 derivative of deoxythymidine (dT) (Cat. No. 10-1039-90, Glen Research, Sterling, VA,). The polynucleotide strand was synthesized by GeneMed Biotechnologies, Inc. About 45 nanomoles of the polynucleotide was dissolved in deionized water and reacted at room temperature with 1 mg of tetramethylrhodamine succimidylester pre-dissolved in one drop of DMSO in 100 mM pH 8.3 sodium bicarbonate buffer. The conjugate was purified under the same conditions as in Example 1. Enzymatic hydrolysis was carried out overnight with a small amount (about 0.5 mg) of micrococcal nuclease in 100 mM pH 8.9 sodium bicarbonate buffer at 37°C. Fluorescence spectra were measured at room temperature with excitation wavelength at 520 nm, and an approximately

two-fold enhancement in fluorescence intensity at 575 nm was obtained after enzymatic hydrolysis.

Example 3 (Comparative)

In this example, quenching (of one of the dye moieties) is achieved by energy transfer rather than dye dimerization.

The following two polynucleotides obtained from GeneMed Biotechnologies, Inc. were used to assay DNase activity by use of fluorescence energy transfer:

A. 5' TTA **X**TA AYT CCG TTT AA 3' **B.** 5' TTA **Z**TA AYT CC**X** TTT AA 3'

where **X** is tetrachlorofluorescien (acceptor), **Y** is fluorescein (donor), and **Z** is the amino-modified C_6 of deoxythymidine (dT) (to allow for attachment of another acceptor, tetramethylrhodamine (TMR), which is pendent from **Z**). In the **A.** configuration, two different dyes, the donor and acceptor, **X** and **Y**, were inserted in the polynucleotide sequence. Unlike Example 2 wherein all of the dye moieties were pendent from the polynucleotide, in this Example the donor and one acceptor were incorporated into the polynucleotide backbone itself. The **B.** configuration is similar to **A.** except a second acceptor (TMR) was pendent from **Z**. When the polynucleotide was intact, the donor and acceptor were within energy transfer distance of each other. The excited-state energy of fluorescein was nonradiatively transferred to tetrachlorofluorescien (and/or tetramethylrhodamine in the **B.** case) causing the donor fluorescence to be quenched. Acceptor fluorescence is not quenched. After enzymatic hydrolysis of the DNA, the donor and acceptor were separated and energy transfer became trivial. The donor fluorescence of compound **A.** after hydrolysis by micrococcal nuclease increased by approximately 30% at about 530 nm on excitation at 490 nm. By visual observation, the fluorescence changed from orange-blue to dark blue, indicating reduced energy transfer efficiency. A similar result was observed for compound **B**.

Example 4

In this example, tetramethylrhodamine was attached to an aliphatic amine linking moiety at the 3' and 5' termini, respectively, of two complementary polynucleotides. Inter-strand hybridization brought the dye moieties into close contact with each other and quenched fluorescence. Enzymatic cleavage at multiple sites along the double-stranded polynucleotide disintegrated the ordered structure to liberate the dye moieties from dimers to monomers, producing a significant increase in fluorescence. This concept is schematically illustrated in Figure 3.

Two complementary 25-mer polynucleotides were synthesized by GeneMed Biotechnologies, Inc.:

NH_2-$(CH2)_3$-5'-**AAC AAA GGA TTA TAG TGG GAA ATC A**-3'
NH_2-$(CH_2)_3$-3'-**TTG TTT CCT AAT ATC ACC CTT TAG T**-5'

Approximately 22.5 nanomoles of each polynucleotide were reacted overnight, separately, with 1 milligram of tetramethylrhodamine (TMR) isothiocyanate in a 100 mM pH 9.0 sodium carbonate buffer at 23°C under agitation (shaking). The dye was first dissolved in one drop of DMSO before mixing with the polynucleotides. A thio-urea bond formed between the isothiocyanate of the TMR moiety and the amine group attached to the polynucleotide. Each reaction mixture was passed through a size-exclusion column (PD-10, Pharmacia Biotech, Inc., Piscataway, NJ), as described in Example 1, to separate labeled species from unreacted dye molecules. The melting temperature, T_m, was calculated based on the percentage of GC pair content in the polynucleotide chain and the ionic strength of the hybridization solution (Catalog Number F123A, Promega Corp., Madison, WI), using the following formula:

$$T_m = 81.5 + 16.6(\log_{10}[Na^+]) + 0.41(\%GC) - (600/N)$$

where N is an integer signifying the number of bases in the polynucleotide chain. This formula predicts the T_m reasonably well for polynucleotides between 14 and

60-70 nucleotides in length. The Na^+ concentration of the Promega hybridization solution is 0.39. Based on this formula, T_m of the above polynucleotide was calculated to be 63.8°C. Hybrids formed between short polynucleotides at 5-10°C below the T_m are reversible and easy to unwind. Therefore, the reaction temperature should be at least 10°C below the T_m. In addition, to prepare stable hybrids, hybridizations with short polynucleotides should be carried out under stringent conditions using high concentrations (0.1-1.0 picomole/ml) of polynucleotides. In this example, hybridization of the two strands bearing fluorescent dyes was carried out overnight at 45°C (19°C below T_m) in a high stringency hybridization solution (the Promega solution). The conjugate solution was shown to have a fluorescence intensity at 575 nm (Ex = 550 nm), approximately six times greater after overnight incubation with micrococcal nuclease (Cat. No. N-3755, Sigma Chemical Co., St. Louis, MO) at 37°C in a 100 mM pH 8.9 bicarbonate buffer than prior to treatment with nuclease. This is illustrated in Figure 4.

Various modifications and alterations of this invention will become apparent to those skilled in the art without departing from the scope and spirit of the invention. It should be understood that this invention is not limited to the illustrative embodiments set forth herein.

CLAIMS

1. A reagent comprising:

at least one enzyme-specific cleavable polynucleotide substrate bearing at least one fluorescent moiety in close proximity to at least one other adjacent fluorescent moiety, said adjacent fluorescent moieties quenching fluorescence in each other, said fluorescent moieties being readily detectable by fluorometric techniques upon cleavage of said substrate.

2. The reagent according to claim 1 wherein said fluorescent moieties comprise fluorescent dye groups.

3. The reagent according to claim 1 or 2 wherein said fluorescent moieties are identical.

4. The reagent according to claim 1 or 2 wherein said polynucleotide substrate comprises about 24 base pairs.

5. The reagent according to claim 1 or 2 wherein said polynucleotide substrate comprises a self-complementary single strand polynucleotide having 3'and 5' termini each bearing a fluorescent moiety.

6. The reagent according to claim 1 or 2 wherein said polynucleotide substrate comprises two complementary hybridized polynucleotide strands each having 3'and 5' termini wherein each terminus of one or both 3'/5' pair(s) bears a fluorescent moiety.

7. The reagent according to claim 1 or 2 wherein said polynucleotide substrate comprises a single strand polynucleotide bearing along its length at least two pendent adjacent fluorescent moieties.

8. The reagent according to claim 7 wherein said adjacent fluorescent moieties are separated from each other by a distance of less than 10 nanometers measured base to base along the polynucleotide backbone.

9. The reagent according to claim 1 or 2 wherein said enzyme-specific cleavable polynucleotide substrate is cleavable by an enzyme selected from the group consisting of DNase and RNase.

10. The reagent according to claim 9 wherein said DNase is a bacterial DNase.

11. The reagent according to claim 2 wherein said fluorescent dye groups comprise two or more dimerized or stacked fluorescent dye groups.

12. The reagent according to claim 2 wherein each of said dye groups comprises a planar portion.

13. The reagent according to claim 2 wherein each of said dye groups is selected from the group consisting of fluorescein, rhodamine, cyanine, and boron heterocyclic dye groups.

14. The reagent according to claim 13 wherein each of said rhodamine dyes is selected from the group consisting of tetramethylrhodamine, X-Rhodamine, and Rhodamine B.

15. A method of biological assay comprising the step of:
combining
(1) a reagent according to claim 1 or 2, and
(2) a test sample potentially containing the specific enzyme being assayed wherein the presence of the specific enzyme being assayed will result in cleavage of the polynucleotide, separation of the fluorescent moieties, and an increase in fluorescence intensity.

16. The method of claim 15 further comprising the step of measuring the increase in fluorescence intensity.

17. The method of claim 16 wherein the increase in fluorescence intensity is measured by a means selected from radiant energy illumination, a fluorescent microscope, a 96-well plate reader, and a flow cytometer.

18. The method of claim 15 wherein said enzyme originates from bacteria.

19. A method of making a reagent comprising an enzyme-specific cleavable polynucleotide bearing fluorescent moieties that quench each other through dye dimerization and that are detectable by fluorometric techniques upon separation, said method comprising the step of:
combining one or more fluorescent compounds bearing one or more fluorescent moieties and one or more reactive groups with enzyme-specific cleavable polynucleotides selected from the group consisting of (1) self-complementary single strand polynucleotides having one or more end groups reactive with one or more of the fluorescent compounds, (2) single strand polynucleotides having within the polynucleotide, at locations other than the termini, two or more moieties having pendent groups reactive with one or more of the fluorescent compounds, and (3) complementary polynucleotides each strand having at least one reactive end group at the 3' and/or 5' termini, respectively, of one or both 3'/5' pair(s) which react with one or more of the fluorescent compounds, such combination taking place under reactive conditions to produce said reagent.

20. The method of claim 19 wherein said compound bearing said fluorescent moieties is selected from the group consisting of fluorescein, rhodamine, cyanine, and boron heterocyclic dye groups.

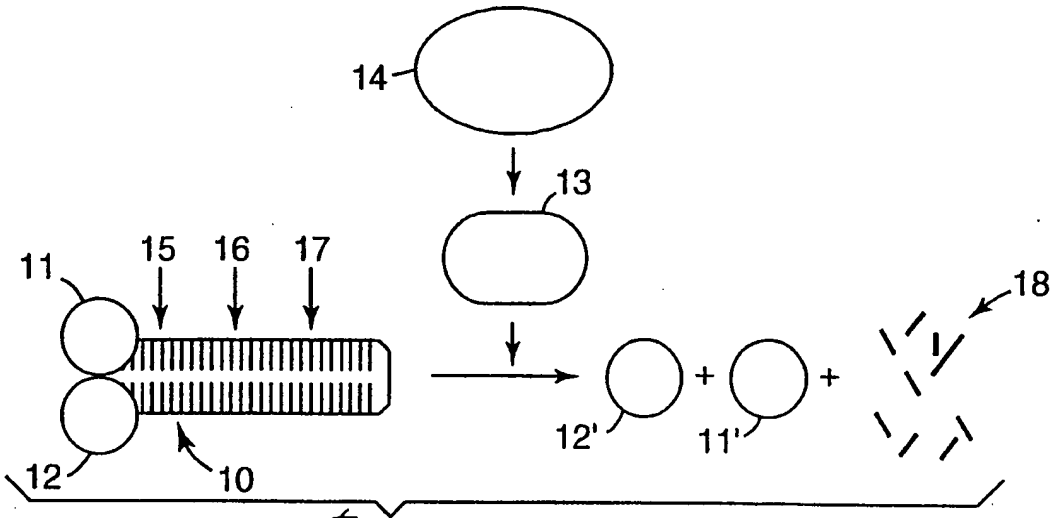

Fig. 1

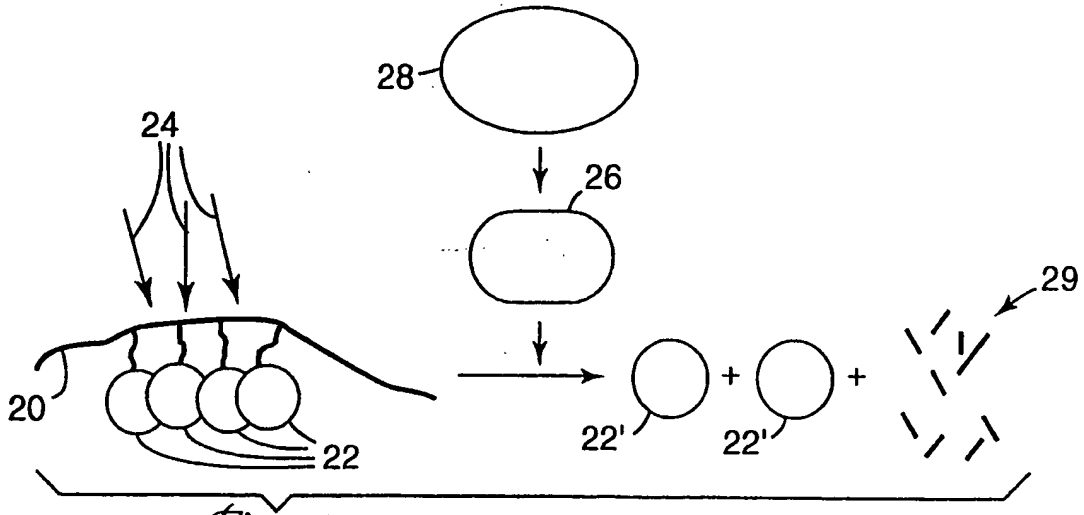

Fig. 2

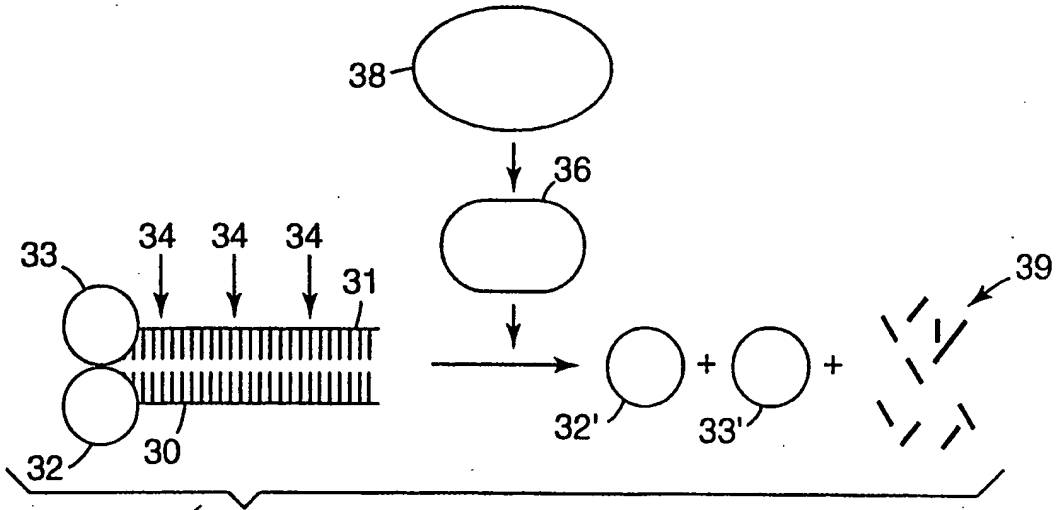
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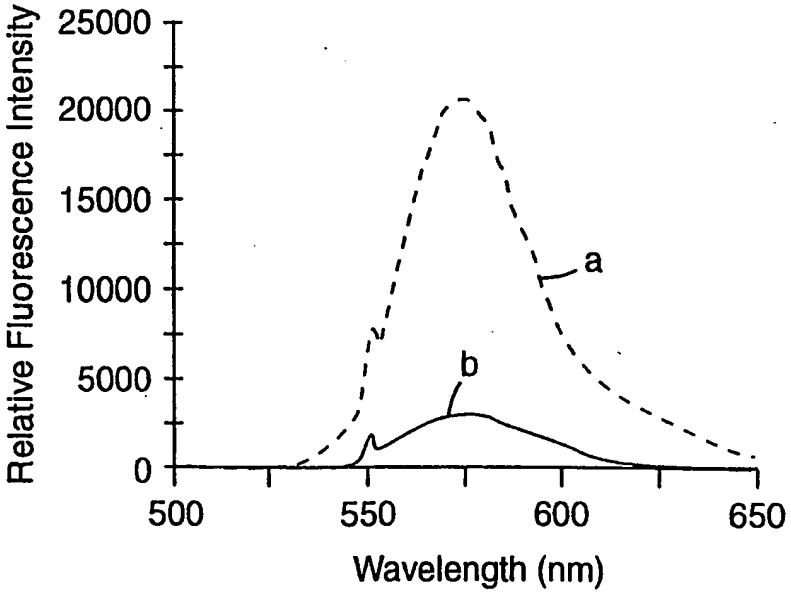
Fig. 3


Relative Fluorescence Intensity
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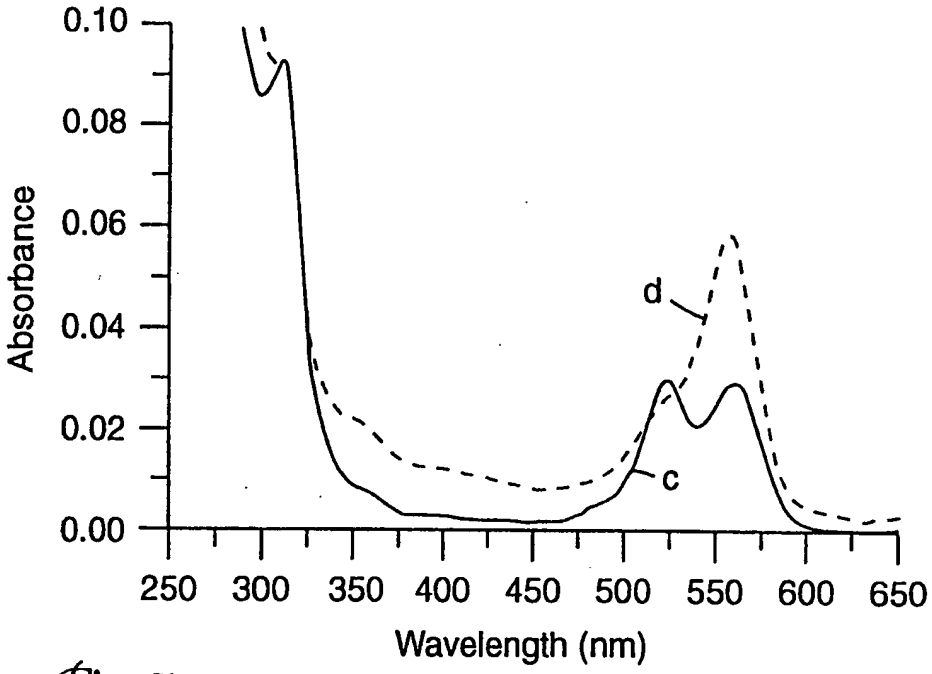
20000
15000
10000
5000
0
500
550
600
650
Wavelength (nm)
a
b

Fig. 4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600
650
Wavelength (nm)

Fig.5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/17311

A. CLASSIFICATION OF SUBJECT MATTER

C 12 Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC6

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C 12 Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0699768 A1 (F. HOFFMANN-LA ROCHE AG) 06 March 1996 (06.03.96), abstract, page 6, lines 34-40, claims 1,2. --	1,2, 12-14, 20
A	US 5700646 A (WOOD, S.J.) 23 December 1997 (23.12.97), fig. 1, claim 1. --	1,2,6, 15,19
A	EP 0428000 A1 (ABBOTT LABORATORIES) 22 May 1991 (22.05.91), abstract, claims 1-4,10. --	1,2,8, 15,16, 19
A	US 5605809 A (KOMORIYA, A. et al.) 25 February 1997 (25.02.97),	1,2,8, 13,14, 16,19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search
18 November 1998

Date of mailing of the international search report
06. 01. 99

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/17311

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	column 2, lines 26-65, column 3, lines 1-11, columns 13,14. ----	

ANHANG

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

ANNEX

to the International Search Report to the International Patent Application No.

ANNEXE

au rapport de recherche international relatif à la demande de brevet international n°

PCT/US 98/17311 SAE 206173

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

La présénte annexe indique les membres de la famille de brevets relatifs aux documents de brevets cités dans le rapport de recherche international visée ci-dessus. Les renseignements fournis sont donnés à titre indicatif et n'engagent pas la responsibilité de l'Office.

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
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